FORM PTO-1390 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE
(REV 11-98)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **1930-A-PCT-US**

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR 1.5): 09/807391

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IB99/01676	**14 October 1999**	**15 October 1998**

TITLE OF INVENTION:
TRANSFORMATION PROCESS

APPLICANT(S) FOR DO/EO/US:
DE RONDE, Jacoba Adriana
CRESS, William

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. X This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C 371.
3. This is an express request to begin national examination procedures (35 U S C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C 371(b) and PCT Articles 22 and 39(1)
4. X A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. X A copy of the International Application as filed (35 U S C. 371 (c) (2))
   a. is transmitted herewith (required only if not transmitted by the International Bureau).
   b. X has been transmitted by the International Bureau.
   c. is not required, as the application was filed in the United States Receiving Office (RO/US)
6. A translation of the International Application into English (35 U.S.C 371(c)(2))
7. X A copy of the International Search Report (PCT/ISA/210).
8. Amendments to the claims of the International Application under PCT Article 19 (35 U S C 371 (c)(3))
   a. are transmitted herewith (required only if not transmitted by the International Bureau).
   b. have been transmitted by the International Bureau.
   c. have not been made; however, the time limit for making such amendments has NOT expired.
   d. have not been made and will not be made.
9. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))
10. X An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. X A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U S C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. X An Information Disclosure Statement under 37 CFR 1 97 and 1.98.
14. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3 28 and 3 31 is included
15. X A **FIRST** preliminary amendment.
16. A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. A substitute specification.
18. A change of power of attorney and/or address letter
19. X Certificate of Mailing by Express Mail
20. Other items or information:

Copy of the WIPO publication No. WO 00/22149

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/807391	PCT/IB99/01676	1930-A-PCT-US

21. The following fees are submitted:.	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,000.00**	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	27 - 20 =	7	x $18.00	**$126.00**
Independent claims	2 - 3 =	0	x $80.00	**$0.00**
Multiple Dependent Claims **(check if applicable)**.			☒	**$270.00**
TOTAL OF ABOVE CALCULATIONS			=	**$1,256.00**
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**.			☒	**$628.00**
SUBTOTAL			=	**$628.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	**$0.00**
TOTAL NATIONAL FEE			=	**$628.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	**$0.00**
TOTAL FEES ENCLOSED			=	**$628.00**
			Amount to be: refunded	$
			charged	$

☒ A check in the amount of **$448.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No **19-0083** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Joseph A. Sebolt, Reg. No. 35,352
SAND & SEBOLT
Aston Park Professional Centre, Suite 194
4801 Dressler Road, NW
Canton, Ohio 44718

SIGNATURE

NAME

Fred H. Zollinger, III

REGISTRATION NUMBER

39,438

DATE April 12, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:	DE RONDE et al
FOR:	**TRANSFORMATION PROCESS**
SERIAL NO.	Not yet known
FILING DATE:	Filed Herewith
ATTORNEY DOCKET NO.	1930-A-PCT-US

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231
BOX NON-FEE AMENDMENT

Dear Sir:

The Applicant respectfully requests the entry of the following preliminary amendment before examination of the application.

In the claims

Please enter the amendments to the claims as shown on the attached sheets entitled "AMENDED CLAIMS - VERSION WITH MARKINGS TO SHOW CHANGES MADE. Applicant requests that each amended claim be substituted for the presently pending claim of the same number. For the Examiner's convenience, a clean version of the amended claims is also provided herewith on the sheets entitled "CLEAN VERSION OF THE AMENDED CLAIMS".

REMARKS

The above-referenced patent application was filed as PCT/IB/01676 on 14 October 1999. The claims were revised under Chapter II of the PCT proceedings, the changes being made to eliminate multiple dependencies and to eliminate omnibus claims. The revisions to the claims may be found on the sheets entitled "AMENDED CLAIMS - VERSION WITH MARKINGS TO SHOW CHANGES MADE." . A clean set of the amended claims may be found on the sheets entitled "CLEAN VERSION OF THE AMENDED CLAIMS".

The claims under consideration in this application are therefore claims 1-26 and claim 28.

If the Examiner believes that a telephone interview would be beneficial to advance prosecution of the instant application to early issue, they are invited to contact the undersigned at the telephone number listed below.

Respectfully submitted at Canton, Ohio this 12th day of April, 2001.

SAND & SEBOLT

By: Fred H. Zollinger, III
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#3
CBwt

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:	DE RONDE et al
FOR:	**TRANSFORMATION PROCESS**
SERIAL NO.	09/807,391
FILING DATE:	04/12/01
ATTORNEY DOCKET NO.	1930-A-PCT-US

SECOND PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231
BOX NON-FEE AMENDMENT

Dear Sir:

Applicant respectfully requests the entry of the following second preliminary amendment prior to examination of the application.

In the claims

Please cancel claims 23-26 and claim 28. The claims therefore remaining in the application are claims 1-22.

REMARKS

When the instant application was filed in the U.S. Patent Office on April 12,

2001, the claims under consideration, after filing of the Preliminary Amendment, were claims 1-26 and claim 28. Applicant's South African agents had instructed Applicant's American agents to file the application with claims 1-22. However, upon review of the documents provided it was unclear as to the status of claims 23-26 and claim 28 in the PCT application. The application had to be filed promptly and consequently Applicant's U.S. agents therefore included claims 23-26 and claim 28 in the national phase application.

It has now become apparent that claims 23-26 and claim 28 were canceled during the PCT international phase. Applicant is therefore requesting cancellation of claims 23-26 and claim 28 to bring the application into parity with the PCT application.

If the Examiner believes that a telephone interview would be beneficial to advance prosecution of the instant application to early issue, they are invited to contact the undersigned at the telephone number listed below.

Respectfully submitted at Canton, Ohio this 30th day of May, 2001.

SAND & SEBOLT

By: Joseph A. Sebolt
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Docket No.1930-A-PCT-US

AMENDED CLAIMS

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Please cancel claims 23-26 and claim 28.

CLEAN VERSION OF AMENDED CLAIMS

1. A method of obtaining transformed or genetically modified plant seed, the method including contacting germinating plant seed with a wetting agent or surfactant and an *Agrobacterium* strain to transform or genetically modify the plant seed.

2. A method as claimed in claim 1, in which the wetting agent or surfactant is a non-oil based wetting agent or surfactant.

3. A method as claimed in claim 1, in which the wetting agent or surfactant includes a polyether polymethyl siloxane copolymer.

4. A method as claimed in claim 1, in which the *Agrobacterium* strain and the wetting agent or surfactant are in the form of an admixture, the wetting agent or surfactant and the *Agrobacterium* strain being present in the admixture in a mass ratio of the wetting agent or surfactant: *Agrobacterium* strain of between 1:99 and 1:10000.

5. A method as claimed in claim 1, in which the germinating plant seed are subjected to vacuum infiltration while they are being contacted with the wetting agent or surfactant and the *Agrobacterium* strain.

6. A method as claimed in claim 5, in which the germinating plant seed are subjected to vacuum infiltration for a period of between 5 minutes and 40 minutes, at a pressure of between 150 Pa (a) and 750 Pa (a).

7. A method as claimed in claim 1, in which the germinating plant seed are contacted with the wetting agent or surfactant and the *Agrobacterium* strain

for a period of between 2 hours and 48 hours, at a temperature of between 15 °C and 35 °C.

8. A method as claimed in claim 1, in which the *Agrobacterium* strain is *Agrobacterium tumefaciens*.

9. A method as claimed in claim 1, in which the *Agrobacterium* strain includes a foreign gene which includes appropriate regulatory sequences so as to be expressed in the cells of a plant which is cultivated from the transformed or genetically modified plant seed.

10. A method as claimed in claim 9, in which the foreign gene confers at least one of disease resistance and drought resistance to the plant which is cultivated from the transformed or genetically modified plant seed.

11. A method as claimed in claim 1, in which the *Agrobacterium* strain includes a plasmid comprising vector pBI121.

12. A method as claimed in claim 9, in which the *Agrobacterium* strain includes a plasmid which includes both said foreign gene and a selection agent resistance gene, the selection agent resistance gene also including appropriate regulatory sequences so as to be expressed in the cells of the plant which is cultivated from the transformed or genetically modified plant seed.

13. A method as claimed in claim 12, in which the selection agent resistance gene codes for antibiotic resistance, thus imparting resistance to an antibiotic selection agent to the plant which is cultivated from the transformed or genetically modified plant seed.

14. A method as claimed in claim 13, in which the antibiotic selection agent is selected from the group consisting of at least one of kanamycin and rifampicin, and in which the selection agent resistance gene is a GUS-intron gene.

15. A method as claimed in claim 1, in which the plant seed is from the family *leguminoseae*.

16. A method as claimed in claim 15, in which the plant seed is soybean seed.

17. A method as claimed in claim 15, in which the plant seed is lupin seed.

18. A method as claimed in claim 1, which includes germinating plant seed at a temperature of between 22 °C and 32 °C, for a period of between 2 days and 5 days, before contacting the germinating plant seed with the wetting agent or surfactant and the *Agrobacterium* strain.

19. A transformed or genetically modified plant seed produced by the method as claimed in claim 1.

20. A transformed or genetically modified plant cultivated from the plant seed as claimed in claim 19.

21. A plant seed produced by the transformed or genetically modified plant as claimed in claim 20.

22. A plant which is the progeny of a transformed or genetically modified plant as claimed in claim 20.

CLEAN VERSION OF AMENDED CLAIMS

1. A method for obtaining transformed or genetically modified plant seed, the method including contacting germinating plant seed with a wetting agent or surfactant and an *Agrobacterium* strain to transform or genetically modify the plant seed.

2. A method as claimed in claim 1, in which the wetting agent or surfactant is a non-oil based wetting agent or surfactant.

3. A method as claimed in claim 1, in which the wetting agent or surfactant includes a polyether polymethyl siloxane copolymer.

4. A method as claimed in claim 1, in which the *Agrobacterium* strain and the wetting agent or surfactant are in the form of an admixture, the wetting agent or surfactant and the *Agrobacterium* strain being present in the admixture in a mass ratio of the wetting agent or surfactant: *Agrobacterium* strain of between 1:99 and 1:10000.

5. A method as claimed in claim 1, in which the germinating plant seed are subjected to vacuum infiltration while they are being contacted with the wetting agent or surfactant and the *Agrobacterium* strain.

6. A method as claimed in claim 5, in which the germinating plant seed are subjected to vacuum infiltration for a period of between 5 minutes and 40 minutes, at a pressure of between 150 Pa (a) and 750 Pa (a).

7. A method as claimed in claim 1, in which the germinating plant seed are contacted with the wetting agent or surfactant and the *Agrobacterium* strain

for a period of between 2 hours and 48 hours, at a temperature of between 15 °C and 35 °C.

8. A method as claimed in claim 1, in which the *Agrobacterium* strain is *Agrobacterium tumefaciens*.

9. A method as claimed in claim 1, in which the *Agrobacterium* strain includes a foreign gene which includes appropriate regulatory sequences so as to be expressed in the cells of a plant which is cultivated from the transformed or genetically modified plant seed.

10. A method as claimed in claim 9, in which the foreign gene confers at least one of disease resistance and drought resistance to the plant which is cultivated from the transformed or genetically modified plant seed.

11. A method as claimed in claim 1, in which the *Agrobacterium* strain includes a plasmid comprising vector pBI121.

12. A method as claimed in claim 9, in which the *Agrobacterium* strain includes a plasmid which includes both said foreign gene and a selection agent resistance gene, the selection agent resistance gene also including appropriate regulatory sequences so as to be expressed in the cells of the plant which is cultivated from the transformed or genetically modified plant seed.

13. A method as claimed in claim 12, in which the selection agent resistance gene codes for antibiotic resistance, thus imparting resistance to an antibiotic selection agent to the plant which is cultivated from the transformed or genetically modified plant seed.

14. A method as claimed in claim 13, in which the antibiotic selection agent is selected from the group consisting of at least one of kanamycin and rifampicin, and in which the selection agent resistance gene is a GUS-intron gene.

15. A method as claimed in claim 1, in which the plant seed is from the family *leguminoseae*.

16. A method as claimed in claim 15, in which the plant seed is soybean seed.

17. A method as claimed in claim 15, in which the plant seed is lupin seed.

18. A method as claimed in claim 1, which includes germinating plant seed at a temperature of between 22 °C and 32 °C, for a period of between 2 days and 5 days, before contacting the germinating plant seed with the wetting agent or surfactant and the *Agrobacterium* strain.

19. A transformed or genetically modified plant seed produced by the method as claimed in claim 1.

20. A transformed or genetically modified plant cultivated from the plant seed as claimed in claim 19.

21. A plant seed produced by the transformed or genetically modified plant as claimed in claim 20.

22. A plant which is the progeny of a transformed or genetically modified plant as claimed in claim 20.

23. A transformation composition which includes, in admixture, an *Agrobacterium* strain and a wetting agent or surfactant.

24. A transformation composition as claimed in claim 23, in which the wetting agent or surfactant is a non-oil based wetting agent or surfactant.

25. A transformation composition as claimed in claim 23 or claim 24, in which the wetting agent or surfactant includes a polyether polymethyl siloxane copolymer, and in which the *Agrobacterium* strain is *Agrobacterium tumefaciens*.

26. A transformation composition as claimed in claim 25, in which the wetting agent or surfactant and the *Agrobacterium* strain are present in a mass ratio of the wetting agent or surfactant: *Agrobacterium* strain of between 1:99 and 1:10000.

28. A transformation composition as claimed in claim 23, substantially as herein described and illustrated.

AMENDED CLAIMS

VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. A method for obtaining transformed or genetically modified plant seed, the method including contacting germinating plant seed with a wetting agent or surfactant and an *Agrobacterium* strain to transform or genetically modify the plant seed.

2. A method as claimed in claim 1, in which the wetting agent or surfactant is a non-oil based wetting agent or surfactant.

3. A method as claimed in claim 1 [or claim 2], in which the wetting agent or surfactant includes a polyether polymethyl siloxane copolymer.

4. A method as claimed in claim 1 [any one of the preceding claims], in which the *Agrobacterium* strain and the wetting agent or surfactant are in the form of an admixture, the wetting agent or surfactant and the *Agrobacterium* strain being present in the admixture in a mass ratio of the wetting agent or surfactant: *Agrobacterium* strain of between 1:99 and 1:10000.

5. A method as claimed in claim 1 [any one of the preceding claims], in which the germinating plant seed are subjected to vacuum infiltration while they are being contacted with the wetting agent or surfactant and the *Agrobacterium* strain.

6. A method as claimed in claim 5, in which the germinating plant seed are subjected to vacuum infiltration for a period of between 5 minutes and 40 minutes, at a pressure of between 150 Pa (a) and 750 Pa (a).

7. A method as claimed in claim 1 [any one of the preceding claims], in

which the germinating plant seed are contacted with the wetting agent or surfactant and the *Agrobacterium* strain for a period of between 2 hours and 48 hours, at a temperature of between 15 °C and 35 °C.

8. A method as claimed in claim 1 [any one of the preceding claims], in which the *Agrobacterium* strain is *Agrobacterium tumefaciens*.

9. A method as claimed in claim 1 [any one of the preceding claims], in which the *Agrobacterium* strain includes a foreign gene which includes appropriate regulatory sequences so as to be expressed in the cells of a plant which is cultivated from the transformed or genetically modified plant seed.

10. A method as claimed in claim 9, in which the foreign gene confers at least one of disease resistance and drought resistance to the plant which is cultivated from the transformed or genetically modified plant seed.

11. A method as claimed in claim 1 [any one of the preceding claims], in which the *Agrobacterium* strain includes a plasmid comprising vector pBI121.

12. A method as claimed in claim 9 [or claim 10], in which the *Agrobacterium* strain includes a plasmid which includes both said foreign gene and a selection agent resistance gene, the selection agent resistance gene also including appropriate regulatory sequences so as to be expressed in the cells of the plant which is cultivated from the transformed or genetically modified plant seed.

13. A method as claimed in claim 12, in which the selection agent resistance gene codes for antibiotic resistance, thus imparting resistance to an antibiotic selection agent to the plant which is cultivated from the transformed or genetically modified plant seed.

14. A method as claimed in claim 13, in which the antibiotic selection agent is selected from the group consisting of at least one of kanamycin and rifampicin, and in which the selection agent resistance gene is a GUS-intron gene.

15. A method as claimed in claim 1 [any and of the preceding claims], in which the plant seed is from the family *leguminoseae.*

16. A method as claimed in claim 15, in which the plant seed is soybean seed.

17. A method as claimed in claim 15, in which the plant seed is lupin seed.

18. A method as claimed in claim 1 [any one of the preceding claims], which includes germinating plant seed at a temperature of between 22 °C and 32 °C, for a period of between 2 days and 5 days, before contacting the germinating plant seed with the wetting agent or surfactant and the *Agrobacterium* strain.

19. A transformed or genetically modified plant seed produced by the method as claimed in claim 1 [any one of claims 1 to 18 inclusive].

20. A transformed or genetically modified plant cultivated from the plant seed as claimed in claim 19.

21. A plant seed produced by the transformed or genetically modified plant as claimed in claim 20.

22. A plant which is the progeny of a transformed or genetically modified plant as claimed in claim 20.

23. A transformation composition which includes, in admixture, an *Agrobacterium* strain and a wetting agent or surfactant.

24. A transformation composition as claimed in claim 23, in which the wetting agent or surfactant is a non-oil based wetting agent or surfactant.

25. A transformation composition as claimed in claim 23 or claim 24, in which the wetting agent or surfactant includes a polyether polymethyl siloxane copolymer, and in which the *Agrobacterium* strain is *Agrobacterium tumefaciens*.

26. A transformation composition as claimed in claim 25, in which the wetting agent or surfactant and the *Agrobacterium* strain are present in a mass ratio of the wetting agent or surfactant: *Agrobacterium* strain of between 1:99 and 1:10000.

[27. A method for obtaining transformed or genetically modified plant seed as claimed in claim 1, substantially as herein described and illustrated.]

28. A transformation composition as claimed in claim 23, substantially as herein described and illustrated.

[29. A new method for obtaining transformed or genetically modified plant seed, a new transformation composition, a new plant seed, or a new plant, substantially as herein described.]

TRANSFORMATION PROCESS

THIS INVENTION relates to a method for obtaining transformed or genetically modified plant seed. It also relates to a transformation composition.

According to one aspect of the invention there is provided a method for obtaining transformed or genetically modified plant seed, the method including contacting germinating plant seed with a wetting agent or surfactant and an *Agrobacterium* strain to transform or genetically modify the plant seed.

The wetting agent or surfactant may be any suitable wetting agent or surfactant which facilitates or enhances penetration and transformation of germinating plant seed by the *Agrobacterium* strain. As hereinafter used, the term "wetting agent" includes surfactants with wetting properties. The wetting agent may be a non-oil based wetting agent, and may include a polyether polymethyl siloxane copolymer. One example of a suitable wetting agent is Break-Thru (available from Goldschmidt Chemical Corporation in Hopewell, USA). It is believed that the active component of Break-Thru is polyether polymethyl siloxane copolymer, Break-Thru being a non-oil wetting agent.

The *Agrobacterium* strain and the wetting agent may be in the form of an admixture or suspension. The wetting agent and the *Agrobacterium* strain may be present in the admixture in a mass ratio of the wetting agent: *Agrobacterium* strain of between 1:99 and 1:10000, e.g. 1:1000.

The germinating plant seed may be subjected to vacuum infiltration while they are being contacted with the wetting agent and the *Agrobacterium* strain. The germinating plant seed may be subjected to vacuum infiltration for a period of between 5 minutes and 40 minutes, e.g. 20 minutes, at a pressure of between 150 Pa (a) and 750 Pa (a), e.g. 585 Pa (a). The vacuum infiltration may be carried out at a temperature of between 15 °C and 35 °C, e.g. 25 °C.

The germinating plant seed may then be contacted with the admixture for a period of between 2 hours and 48 hours, e.g. 24 hours, at a temperature of between 15 °C and 35 °C, e.g. room or ambient temperature.

The *Agrobacterium* strain may be any suitable strain such as *Agrobacterium tumefaciens*, for example, *Agrobacterium tumefaciens* strain LBA4404 deposited at Centraalbureae voor Schimmel-cultures (CBS) in the Netherlands under No. CBS 191.83 on 24 February 1983.

The germinating plant seed may be transformed by the introduction of foreign DNA via the *Agrobacterium* strain. Thus, the germinating plant seed may be transformed by exposing or contacting the germinating plant seed with a culture of *Agrobacterium*, said *Agrobacterium* strain being transformation competent and including a construct comprising a foreign gene, the foreign gene including appropriate regulatory sequences so as to be expressed in the cells of a plant which is cultivated from the transformed or genetically modified plant seed.

The foreign gene may be any suitable gene, such as a foreign gene which confers disease resistance and/or drought resistance to the plant which is cultivated from the transformed or genetically modified plant seed.

The *Agrobacterium* strain may include a suitable plasmid to facilitate transformation of the plant seed.

The plasmid may include a vector, such as vector pBI121.

The method may include inducing further growth of the transformed plant seed and selecting for a transformant in the presence of a selecting agent. The *Agrobacterium* strain may include a plasmid which includes both said foreign gene and a selection agent resistance gene, the selection agent resistance gene also including appropriate regulatory sequences so as to be expressed in the cells of the plant which is cultivated from the transformed or genetically modified plant seed.

The selection agent resistance gene may code for antibiotic resistance, thus imparting resistance to an antibiotic selection agent to the plant which is cultivated from the transformed or genetically modified plant seed. The antibiotic selection agent may be selected from the group consisting of at least one of kanamycin and rifampicin, and the selection agent resistance gene may be a GUS-intron gene.

It will be appreciated that any suitable plant seed may be transformed using the method as herein described. The plant seed may be from the family *Leguminoseae* or any other dicotyledonous plant, for example, soybean or lupin seed. If soybean seed is used, the soybean seed may be allowed to germinate until it has a small plumule, easily removable seed coat and cotyledons which are not appressed against each other before the germinating soybean seed is contacted with the wetting agent and the *Agrobacterium* strain. If lupin seed

is used, the lupin seed may be allowed to germinate until the plumule is between 10 - 20 mm in size, before the germinating lupin seed is contacted with the wetting agent and the *Agrobacterium* strain.

The method may include germinating plant seed at a temperature of between 22 °C and 32 °C, e.g. about 29 °C, for a period of between 2 days and 5 days, e.g. about 4 days before contacting the germinating plant seed with the wetting agent and the *Agrobacterium* strain.

The invention extends to a transformed or genetically modified plant seed produced by the method as herein before described, to a transformed or genetically modified plant cultivated from said plant seed, to a plant seed produced by said transformed or genetically modified plant, and to a plant which is the progeny of said transformed or genetically modified plant.

The transformed or genetically modified plant may comprise cells which comprise in their genome at least one preselected foreign gene which produces a foreign cellular product encoded by the foreign gene. The foreign gene may code for at least one of disease resistance and drought resistance.

According to another aspect of the invention, there is provided a transformation composition which includes, in admixture, an *Agrobacterium* strain and a wetting agent.

The wetting agent may be a non-oil based wetting agent. The wetting agent may include a polyether polymethyl siloxane copolymer, and the *Agrobacterium* strain may be *Agrobacterium tumefaciens.*

The wetting agent and the *Agrobacterium* strain may be present in a mass ratio of the wetting agent: *Agrobacterium* strain of between 1:99 and 1:10000, e.g. 1:1000.

The invention will now be described by way of non-limiting example, with reference to the following Figures and examples of methods of transforming plant seed, in accordance with the invention.

Figure 1A shows X-GLUC histochemical localization of GUS enzyme activity in GUS transformed soybean cultivar Carnia 2233 leaf tissue;

Figure 1B shows X-GLUC histochemical localization of GUS enzyme activity in GUS transformed soybean cultivar Carnia 2233 root tissue;

Figure 2 shows X-GLUC histochemical localization of GUS enzyme activity in GUS-INT transformed soybean cultivar Carnia 2233 stomata;

Figure 3 shows the effect of mannitol stress on proline synthesis in third generation transformed soybean cultivar Carnia 2233 with an antisense P5CR construct.

Figure 4 shows a woodenbox screening of control soybean cultivar Carnia 2233 (without *Arabidopsis* P5CR gene) compared to transformed soybean cultivar Carnia 2233 (containing P5CR gene in antisense orientation);

Figure 5A shows the effect of higher copy number of P5CR gene on chlorophyll fluorescence in transformed soybean cultivar Ibis plants (normalised normal plant = NN; normalised transformant antisense orientation = NT AS);

Figure 5B shows the effect of higher or lower copy number of P5CR gene on chlorophyll fluorescence in transformed Ibis plants (normalised transformed antisense = NT AS; normalised transformed sense = NT S; normalised normal plant = NN); and

Figure 6 shows transformed lupin leaves of initial transformed seed, together with first generation seed compared to control plants leaves and seeds.

EXAMPLE 1

Agrobacterium tumefaciens strain LBA4404 containing a CaMV 35S GUS gene (pBI121) was cultured at 27°C in 100 ml Luria-Bertani broth (LB) pH 7.00 supplemented with 150μg/ml rifampicin and 100μg/ml kanamycin until an

absorbence of A_{600} = 0.5 was obtained. 0.01 mg/ml Acetosyringone was added to the *Agrobacterium tumefaciens* culture approximately 24 hours before transformation of plant seeds was carried out. The *Agrobacterium tumefaciens* culture was centrifuged at 10000 rpm for 20 minutes at a temperature of 10°C. Flocculation was avoided or inhibited by dilution of the *Agrobacterium tumefaciens* culture with distilled water to obtain a ratio of *Agrobacterium tumefaciens* : distilled water of 1:4. 0.1% Break-Thru (a wetting agent) (obtainable from Goldschmidt Chemical Corporation was added to the diluted *Agrobacterium tumefaciens* culture.

Soybean seed (Carnia 2233) was sterilised for 5 minutes in 3.5% (v/v) NaOCl, and then washed in sterile water before being germinated on sterile 0.8% water agar at a temperature of 29°C for a period of 2 to 5 days. The germinating soybean seeds were then sorted and soybean seeds having a small plumule, easily removable seed coat and cotyledons which were not appressed against each other, were selected. The selected germinating or germinated soybean seeds were then contacted with the *Agrobacterium*/ wetting agent suspension and vacuum infiltrated for 20 minutes under a pressure of 78 millitorr. After vacuum infiltration, the germinating soybean seeds were then incubated for a further period of 24 hours in contact with the *Agrobacterium*/wetting agent suspension solution at room or ambient temperature to obtain transformed soybean seeds. The transformed soybean seeds were then planted in a soil mixture comprising soil, sand, vermiculite (5:5:3) and grown in a greenhouse to obtain transformed soybean plants. Percentage success rate of transformation was determined by detecting GUS-gene activity using a fluorometric and a histochemical assay.

When the transformed plants had developed a second set of leaves, a leaf from the main apex was tested for expression of the GUS gene. A fluorometric GUS assay (Jefferson, R.A., Kavanagh, T.A. & Bevan, M.W. 1987). GUS fusion ß - glucuronidase as a sensitive and versatile gene fusion marker in

higher plants. *EMBO* J. 6 (13): 3901-3907 was used for screening of all explants for the expression of glucuronidase enzyme. 100 µl sodium phosphate buffer was pipetted into the wells of a microtiter plate. The assay buffer contained 50 mM $NaPO_4$ (pH7.00), 10mM EDTA, 0.1% (v/v) Triton X-100, 10mM mercapto ethanol and 2 M methyl umbilliferyl glucuronide (Sigma). Small pieces of plant tissue were crushed in this buffer and incubated overnight at a temperature of 37°C in the dark. Reactions were visualised on a long wave length UV light box. A histochemical assay was also performed in testing putative transformants. Plant tissue, which tested positive in the fluorescence assay, was incubated overnight at a temperature of 37°C in the dark in a histochemical staining solution. The staining solution contained 50mM $NaPO_4$ (pH7.00), 0.1% (v/v) Triton X-100, 1.04 mM X-Gluc and 0.5% (v/v) DMSO. The plant tissue was subsequently washed in FAA for 10 minutes, followed with a wash in 50% ethanol. The tissue was dehydrated with 100% ethanol and hydrated slowly up to 100% H_2O.

Transformed soybean plants (TO = plants obtained from initial transformed seed; T1, T2 and T3 = plants obtained from self pollinated, first to third generation, transformed plants) were tested for GUS activity and positive results were obtained indicating transformation. GUS expression patterns of soybean cultivar Carnia 2233 transformed with the CaMV 35S gene, were thus observed in leaf (Figure 1A) and root (Figure 1B) tissue of the plant. Plants tested positive for the GUS gene up to the third generation.

To verify the insertion of the GUS gene in the plant genome, molecular analysis, i.e. PCR reaction as well as a Southern blot procedure (Maniatis, T. Fritsch, E.F. & Sambrook, J., 1982. *Molecular Cloning : A Laboratory Manual.* Cold Spring Harbour Laboratory Press, Cold Spring Harbour, H.Y.), were conducted with GUS and NPTII specific primers. PCR and Southern blot observations indicated the presence of the GUS gene in the soybean genome. Plants tested positive up to the third transformant generation.

EXAMPLE 2

In another embodiment of the invention using essentially the same methods and techniques as described in Example 1, soybean seed (Carnia 2233) was transformed using an *Agrobacterium* strain LBA4404 containing a p35S GUS INT gene. It will be appreciated that a GUS-intron gene has the ability to discriminate between prokaryotic organisms such as *Agrobacterium tumefaciens* and eukaryotic organisms such as plants. Only plant tissue containing the GUS-INT gene turns blue in colour in association with a histochemical assay. Any *Agrobacterium* possibly still present in the plant tissue does not turn blue in colour. Figure 2 shows transformed plant cells which have been stained with X-GLUC, indicating that the soybean seed was transformed with the GUS-intron gene and that the GUS activity does not arise from endogenous *Agrobacteria*.

EXAMPLE 3

In a further embodiment of the invention using essentially the same methods and techniques described above for Example 1, soybean seed (Carnia 2233) was transformed with an antisense construct of a proline gene (L-D^1-pyrroline-5-carboxylate reductase = P5CR). The proline gene P5CR was obtained from N. Verbruggen, Laboratory of Genetics, University of Gent, Belgium. The P5CR gene was cloned in antisense orientation into plasmid HB101pMA445, containing a heat inducible promoter which was subsequently triparental mated to A*grobacterium tumefaciens* (Armitage, P. 1988. Transformation of dicotyl plant cells using the Ti plasmid of *Agrobacterium tumefaciens* and Ri plasmid of *A. rhizogenes.* In: Plant Genetic Engineering and Gene Expression: A laboratory Manual. Draper, J., Scott, R., Armitage, P. and Welden, R. (Eds). Blackwell Scientific Publications, Oxford. pp69-160). The construct included a kanamycin resistant gene which can be used in screening of transformants.

Putative transformed seed (with the P5CR gene) were tested for the presence of kanamycin resistance to indicate transformation. Third generation transformed soybean seed and untransformed soybean seed were tested for germination viability on agar plates supplemented with kanamycin. It was noted that all the seeds germinated on agar plates with 0mg/ℓ kanamycin (see Table 1 below). It was noted that as the concentration of kanamycin in the agar plates was increased, the percentage of germinating plants decreased. Some of the seed initially started to germinate for a short period before dying off and some of the germinating plants showed deformities. At the highest concentration of kanamycin in the agar plates, none of the untransformed soybean seeds germinated whereas in contrast 37.5% of the transformed soybean seeds were able to germinate successfully. The plants which germinated successfully were planted in a greenhouse and the results indicated that the transformed plants which germinated were most probably transformed with the P5CR gene.

Table 1: Germination of transformed soybean seed and untransformed control soybean seed on kanamycin supplemented agar plates

	0 mg/ℓ km	25 mg/ℓ km	35 mg/ℓ km	50mg/ℓ km
Untransformed soybean seed	100%	0%	0%	0%
Transformed soybean seed	100%	80%	75%	37.5%

km - kanamycin

The untransformed control plants and the transformed plants were subjected to a variety of tests including different physiological techniques e.g. proline accumulation and anatomical techniques e.g. woodenbox screening to compare the untransformed control plants with the transformed plants under drought and osmotic stress.

Inactivation of the P5CR gene resulted in decreased proline synthesis. The application of a mannitol stress test resulted in the untransformed control plants showing a significant increase in proline concentration whereas the antisense transformed plants displayed a significant decrease in proline concentration, indicating that the P5CR gene had been inactivated in the transformed plants and the transformed plants were unable to synthesise proline in response to the osmotic stress test.

The results of the tests indicate that antisense transformed plants were more drought sensitive than untransformed control plants, which indicates an underexpression of the P5CR gene, as a result of the antisense construct.

Control plants and fourth generation antisense transformed plants were subjected to a woodenbox experiment. Seed was planted in a box and watered until all plants reached the six leaf stage. At this stage the promoter was activated and drought stress was applied. The control plants survived a six day drought stress significantly more than most of the antisense transformed plants which died. The results indicate that soybean plants were successfully transformed with the antisense construct, as the transformed plants were unable to survive a drought stress test with a lower copy number of the proline gene (see Figure 4).

EXAMPLE 4

In order to test the reproducibility of the transformation method further, another soybean cultivar Ibis was transformed with a P5CR gene in sense and antisense direction. Essentially the same methods and techniques described above for Example 1 were used to transform the soybean cultivar Ibis with the P5CR gene. PCR demonstrated that transformation of the Ibis cultivar was successful. Sense and antisense transformants were evaluated physiologically to confirm transformation as well as the effect of the two different constructs on

Ibis. Detection of Ibis transformants was conducted using a chlorophyll fluorescence test. Changes in the photo system II activity of photosynthesis during drought stress can be analysed by measuring changes in different parameters of chlorophyll fluorescence.

The results of the chlorophyll fluorescence test are shown graphically in Figures 5A and 5B, in which the following abbreviations are used:

yO	-	A measurement of the ability of a trapped excition to move an electron into electron transport.
ABS/RC	-	The absorption flux of photons per photosystem II (PSII) reaction centre (RC).
ABS/CSm	-	The absorption flux of photons per PSII cross section (CS).
TRo/RC	-	The rate at which an excition is trapped by the RC.
ETo/RC	-	The electron transport per RC.
DIo/RC	-	The energy flux, which is wasted per RC as heat or transfer to other systems.
RC/CSo	-	Reaction centre per cross section.
RC/CSm	-	An indication of the number of active RCAEs (density) per CS.
TRo/CSm	-	The rate at which an exciton is trapped by the CS.
ETo/CSm	-	The electron transport per CS.
DIo/CSm	-	The energy flux, which is wasted per CSm as heat or transfer to other systems (Strasser B.J and Strasser R.J. 1995. Measuring fast fluorescence transients to address environmental questions: the JIP test. In: Photosynthesis: from light to biosphere (P.Mathis. Ed.) Vol. 5: 977-980. Kluwer Academic Publishers, Dordrecht. ISBN 0-7923-3862-6).

The antisense transformed plants (see Figure 5B) were subjected to stress tests which resulted in the antisense transformed plants increasing active

reaction centres, absorption, trapping and electron transport per cross section in compensating for stress imposed on the antisense transformed plants, whereas in contrast the sense transformed plants (see Figure 5A) when subjected to stress tests shut down certain reaction centres and there was a decrease in trapping, absorption and electron transport. The results indicated that sense transformed plants are better able to survive the stress tests as compared to the antisense transformed plants and control plants. Wasted energy per cross section was lower in the sense transformed plants than in the antisense transformed plants. The sense transformed plants contain a higher P5CR copy number and are therefore able to use energy more efficiently than the antisense transformed plants having a lower P5CR copy number. It appears that the sense transformed plants are more drought tolerant whereas the antisense transformed plants are more drought sensitive. These tests indicated that transformation of Ibis was successful.

EXAMPLE 5

In a further embodiment of the invention using essentially the same methods and techniques described above for Example 2, *Lupinus albus* seed (cultivar Esta) was transformed with *Agrobacterium* strain LBA4404 containing a p35S GUS INT gene. Figure 6 shows transformed lupin leaves of initial transformed seed, together with first generation seed compared to control plants, leaves and seeds. The transformed leaves and seed demonstrate blue colouring representing GUS-gene activity. This indicates that lupin was succesfully transformed using the method according to the invention and that the GUS INT gene was successfully transferred at least to the first generation.

SUMMARY

The methods in accordance with the invention resulted in a transformation success rate of approximately 35% of the soybean seeds. This

is a relatively high transformation success rate in that conventional techniques usually only have a transformation success rate of less than 5%. By inserting a foreign gene or genes into a plasmid in *Agrobacterium tumefaciens*, the soybean seed may be transformed with the foreign gene. The foreign gene is then included in the cells of a soybean plant which grows from the transformed soybean seed and may then be inherited by its progeny.

Advantages of the invention are that the methods are relatively easy to carry out and relatively inexpensive compared to conventional transformation procedures and techniques. As no tissue culture steps are used in the methods according to the invention, it is believed that there will be little or no loss of genetic traits which would usually occur as a result of somatic mutations. The methods in accordance with the invention can be used for transforming any suitable plant seed with genes of interest or agricultural usefulness, for example, drought resistant or disease resistant genes. The methods in accordance with the invention can also be used for producing transgenic plants of other species where routine tissue culture procedures have not yet been established.

CLAIMS:

1. A method for obtaining transformed or genetically modified plant seed, the method including contacting germinating plant seed with a wetting agent or surfactant and an *Agrobacterium* strain to transform or genetically modify the plant seed.

2. A method as claimed in claim 1, in which the wetting agent or surfactant is a non-oil based wetting agent or surfactant.

3. A method as claimed in claim 1 or claim 2, in which the wetting agent or surfactant includes a polyether polymethyl siloxane copolymer.

4. A method as claimed in any one of the preceding claims, in which the *Agrobacterium* strain and the wetting agent or surfactant are in the form of an admixture, the wetting agent or surfactant and the *Agrobacterium* strain being present in the admixture in a mass ratio of the wetting agent or surfactant: *Agrobacterium* strain of between 1:99 and 1:10000.

5. A method as claimed in any one of the preceding claims, in which the germinating plant seed are subjected to vacuum infiltration while they are being contacted with the wetting agent or surfactant and the *Agrobacterium* strain.

6. A method as claimed in claim 5, in which the germinating plant seed are subjected to vacuum infiltration for a period of between 5 minutes and 40 minutes, at a pressure of between 150 Pa (a) and 750 Pa (a).

7. A method as claimed in any one of the preceding claims, in which the germinating plant seed are contacted with the wetting agent or surfactant and

the *Agrobacterium* strain for a period of between 2 hours and 48 hours, at a temperature of between 15 °C and 35 °C.

8. A method as claimed in any one of the preceding claims, in which the *Agrobacterium* strain is *Agrobacterium tumefaciens*.

9. A method as claimed in any one of the preceding claims, in which the *Agrobacterium* strain includes a foreign gene which includes appropriate regulatory sequences so as to be expressed in the cells of a plant which is cultivated from the transformed or genetically modified plant seed.

10. A method as claimed in claim 9, in which the foreign gene confers at least one of disease resistance and drought resistance to the plant which is cultivated from the transformed or genetically modified plant seed.

11. A method as claimed in any one of the preceding claims, in which the *Agrobacterium* strain includes a plasmid comprising vector pBI121.

12. A method as claimed in claim 9 or claim 10, in which the *Agrobacterium* strain includes a plasmid which includes both said foreign gene and a selection agent resistance gene, the selection agent resistance gene also including appropriate regulatory sequences so as to be expressed in the cells of the plant which is cultivated from the transformed or genetically modified plant seed.

13. A method as claimed in claim 12, in which the selection agent resistance gene codes for antibiotic resistance, thus imparting resistance to an antibiotic selection agent to the plant which is cultivated from the transformed or genetically modified plant seed.

14. A method as claimed in claim 13, in which the antibiotic selection agent is selected from the group consisting of at least one of kanamycin and rifampicin, and in which the selection agent resistance gene is a GUS-intron gene.

15. A method as claimed in any and of the preceding claims, in which the plant seed is from the family *leguminoseae.*

16. A method as claimed in claim 15, in which the plant seed is soybean seed.

17. A method as claimed in claim 15, in which the plant seed is lupin seed.

18. A method as claimed in any one of the preceding claims, which includes germinating plant seed at a temperature of between 22 °C and 32 °C, for a period of between 2 days and 5 days, before contacting the germinating plant seed with the wetting agent or surfactant and the *Agrobacterium* strain.

19. A transformed or genetically modified plant seed produced by the method as claimed in any one of claims 1 to 18 inclusive.

20. A transformed or genetically modified plant cultivated from the plant seed as claimed in claim 19.

21. A plant seed produced by the transformed or genetically modified plant as claimed in claim 20.

22. A plant which is the progeny of a transformed or genetically modified plant as claimed in claim 20.

23. A method for obtaining transformed or genetically modified plant seed as claimed in claim 1, substantially as herein described and illustrated.

24. A new method for obtaining transformed or genetically modified plant seed, a new plant seed, or a new plant, substantially as herein described.

Figure 1A

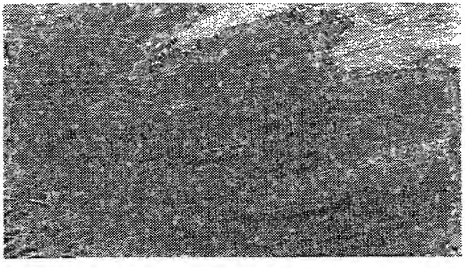
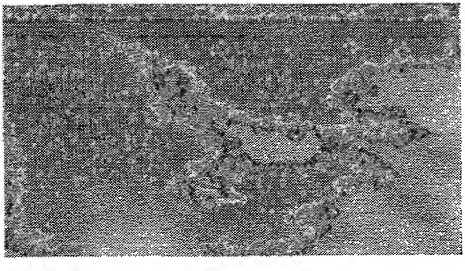


Figure 1B



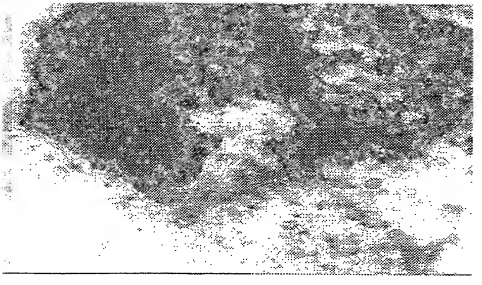


Figure 2

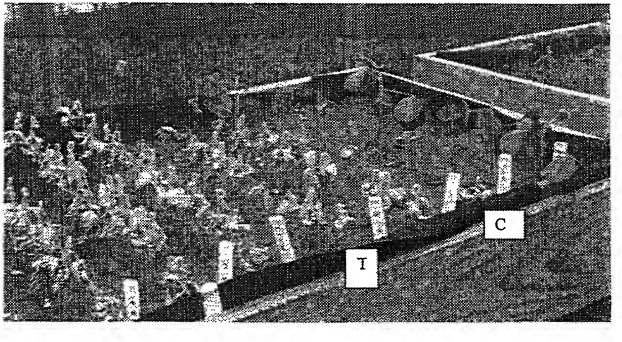


Figure 4

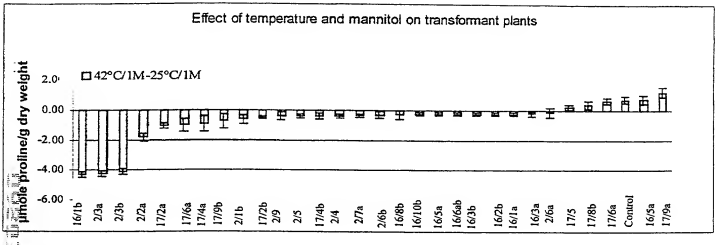


Figure 3

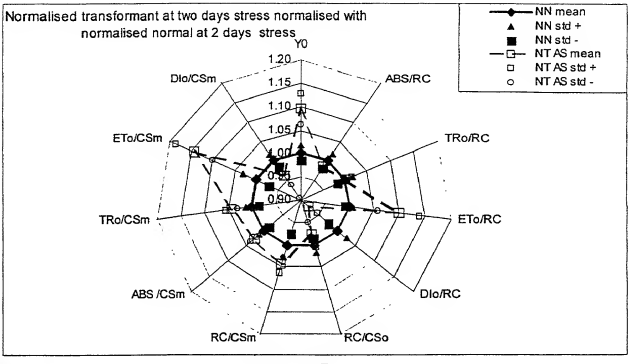


Figure 5A

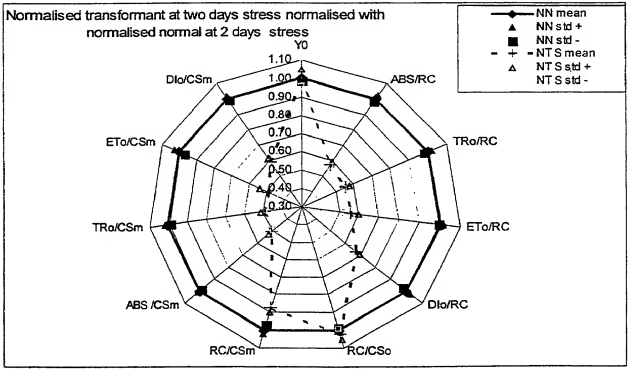


Figure 5B

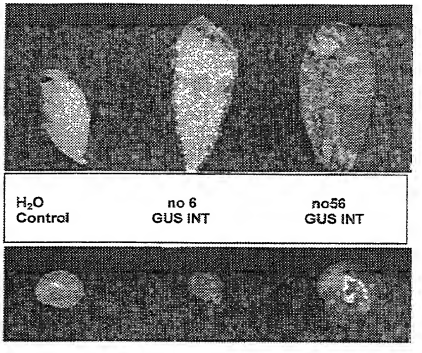


Figure 6

Docket No.
1930-A-PCT-US

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

TRANSFORMATION PROCESS

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **14 October 1999** as United States Application No. or PCT International Application Number **PCT/IB99/01676**

and was amended on **27 September 2000**

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
ZA 98/9427	**South Africa**	**15 October 1998**	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/IB99/01676	**14 October 1999**	**Pending**
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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